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TITLE: Modeling Aggressive Medulloblastoma Using Human-Induced Pluripotent Stem Cells

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CONTRACTING ORGANIZATION: Hugo W. Moser Research Institute at Kennedy Krieger, Inc., Baltimore, MD 21205

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14. ABSTRACT

The goals of this project are to model MYC-driven medulloblastoma by using human stem cells and to further study the transcriptional mechanisms underlying the tumor formation process of MYC-driven medulloblastoma. Our current results strongly support that neural progenitors induced from human induced pluripotent stem cells by Atoh1 induction can be efficiently transformed by MYC oncogene to form aggressive brain tumors that recapitulate human MYC-driven group 3 medulloblastoma. We further show that it is feasible to establish neurosphere cultures from these medulloblastoma tumors to enrich brain tumor stem cells. This neurosphere culture model are suitable for studying gene functions and also testing novel therapies. Overall, this new MYC-driven medulloblastoma model we established provides a reliable model for developing and testing potential therapies for this highly aggressive pediatric brain tumors. It is also feasible to use this medulloblastoma model derived from human stem cells to perform personalized drug discovery and investigate the role of individual's distinctive genetic background in carcinogen sensitivity and medulloblastoma susceptibility.

15. SUBJECT TERMS

none provided

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Introduction:

Brain tumors are the most common cause of childhood oncological death, and medulloblastoma (MB) is the most common malignant pediatric brain tumor. MB patients can be classified into four subgroups, and patients with Group 3 MB are more likely to have aggressive tumors and the worst prognosis. Group 3 MB is characterized by amplification and overexpression of MYC oncogene, herein referred to as MYC-driven MB. Modeling MYC-driven MB is critical for developing and testing potential therapies for this highly aggressive MB. Here, we will use neural cells derived from human induced pluripotent stem cells (iPSCs) to establish MYC-driven MB model. This model will be used to identify the gene network regulated by MYC. We will also study the interaction between MYC and Atoh1, another essential transcription factor for MB development. If successful, our proposed research will establish a new model of human MYC-driven MB, which can be immediately utilized to test existing drugs or develop drugs for patients with Group 3 MYC-driven MB. This MB model derived from human stem cells will provide a personalized tumor model for drug discovery and investigating the role of individual's distinctive genetic background in carcinogen sensitivity and MB susceptibility. By studying this human MYC-driven MB model, we will further provide novel insights into MYC-regulated gene network and the cross-talk between MYC-regulated and Atoh1-regulated gene networks. Understanding the molecular signaling that drives this aggressive tumor will lead to the identification of new therapeutic targets for developing effective treatment to cure this disease. We anticipate our proposed research will generate a preclinical MB tumor model in the short term and show long-term clinically-relevant impact on pediatric brain tumor research through in-depth study of this model.

Keywords:

brain tumors, medulloblastoma, pluripotent stem cells, MYC, transcription factors, high-throughput sequencing.

Accomplishments:

Specific Aim 1: To establish a human-iPSC-derived MYC-driven MB model and test the effects of Atoh1 expression on tumor initiation and growth in this MB model.

Major Task 1: Derive NPCs and NSCs from human iPSCs and check marker expression. (Month: 1-4)

We have generated Atoh1+NPCs and CD133+ NSCs from human iPSCs and applied multiple markers (e.g. CD133, Atoh1, TUJ1, Sox2, Nestin) to confirm the cell identity of these NPCs and NSCs.

Milestone achieved: These cells are ready for testing MYC-driven tumor formation in immunodeficient mice.

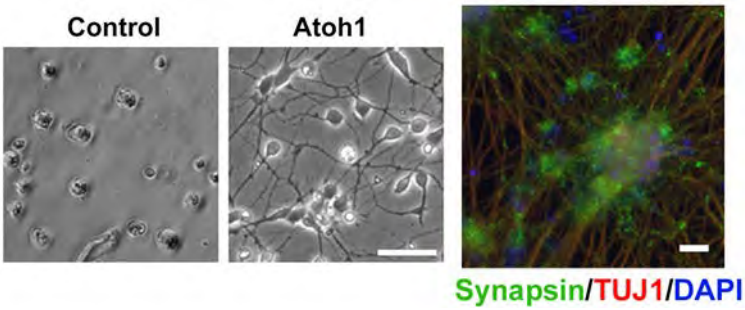

Fig. 1: Atoh1 induces highly efficient differentiation of human iPSCs into neurons. Control iPSCs without Atoh1 induction did not survive in neuron culture condition. Atoh1-induced neurons express neuron markers (Synapsin and TUJ1).

Major Task 2: Compare the ability of human-iPSC-derived Atoh1+ NPCs and CD133+ NSCs to generate MYC-driven MB tumors in Mice.

Subtask 1: Obtain ACURO approval for animal use. (Month: 1-4)

Mileston achieved: The animal use protocol for this project has been approved by Johns Hopkins University Animal Care and Use Committee and USAMRMC Animal Care and Use Review Office (ACURO).

Subtask 2: Perform cell infection and transplantation. (Month: 5-7)

Subtask 3: Monitor animal survival and collect tumors from animals. (Month: 8-11)

Subtask 4: Pathological analysis. (Month: 11-13)

We infected iPSC-derived Atoh1-induced NPCs with lentiviruses expressing a stabilized form of MYC (MYCT58A) and dominant-negative p53 (DNp53). Transgene expression has been validated by western blotting. We transplanted these lentivirus-infected NPCs into the cerebellum of immunodeficient NSG mice.

We monitored animal survival and found aggressive tumor growth in these mice (Fig. 2A, median survival=33).

We also collected tumors for pathological analysis. These MYC-driven tumors were comprised of poorly differentiated, medium to large size cells which showed nuclear molding, prominent nucleoli (Fig. 2B) and numerous mitotic (Ki67+) and apoptotic (Cleaved-Caspase-3+) cells (Fig. 2C). These tumors also expressed early neuronal lineage marker (β-tubulin III, Fig. 2C). All these features closely mimic human Group 3 MB.

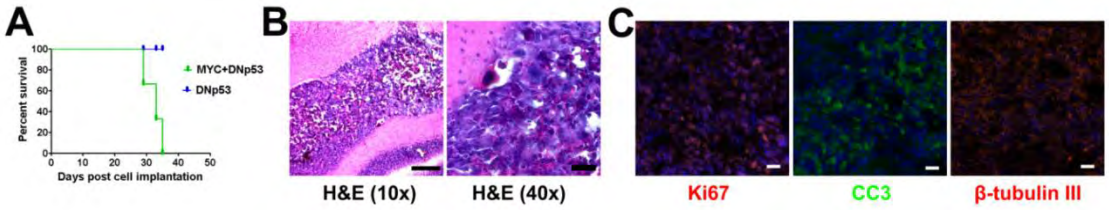

Fig. 2: Figure 2. MYC-driven MB tumors from human Atoh1+ NPCs.
(A) Survival curve of mice receiving 5,000 Atoh1+ NPCs infected with MYC+DNp53 or DNp53 alone. (B) H&E staining of tumor sections generated from Atoh1+ NPCs infected with MYC+DNp53. (Bar:100μm and 20μm in 10x and 40x images, respectively) (C) Tumor sections were stained with antibodies for Ki67, Cleaved Caspase 3 (CC3) and β-tubulin III. (Nuclei were counterstained with DAPI. Bar: 20μm)

We have successfully established neurosphere cultures from MYC-driven MB tumors from human Atoh1+ NPCs (Fig. 3A). These neursphere cultures can be maintained in culture for >20 generations without losing their sphere-forming capability (Fig. 3B), an in vitro hallmark of stem cell self-renewal. These MYC-driven MB tumor derived neurospheres show expression of neuronal lineage marker (TUJ1), but not glial (GFAP) lineage marker (Fig. 4). They express markers for brain tumor stem cells, such as CD133 (>20%, Fig. 3C), Nestin and Sox2 (Fig. 4). They also express markers for human group 3 MB (LGR5 and NPR3, Fig. 4).

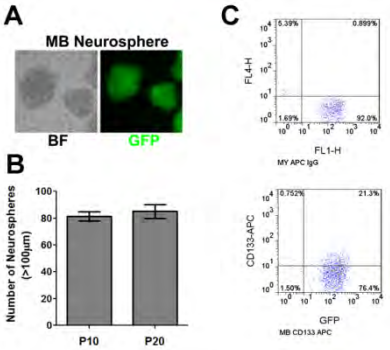

Fig. 3 MYC-driven MB tumor derived neurospheres (A) can be maintained in vitro with >20 passages without losing sphere forming capability (B). CD133+ cells (>20%) are detected in these neurospheres by flow cytometry (C).

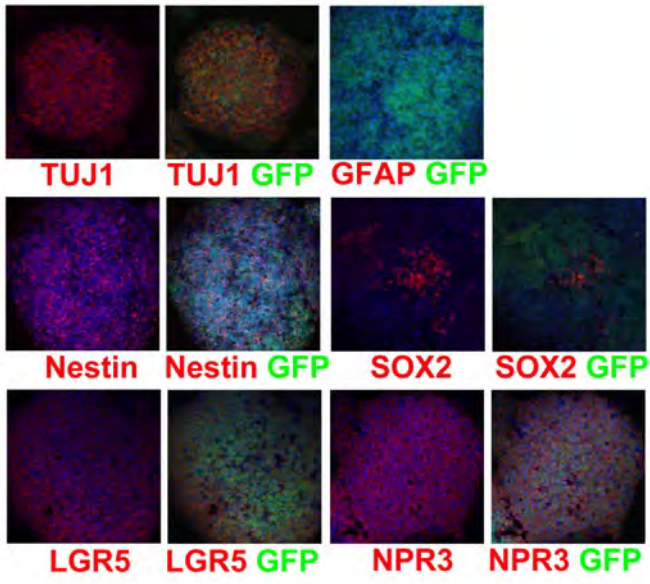

Fig. 4 MYC-driven MB tumor derived neurospheres were immunostained for various markers for neuronal lineage marker (TUJ1), glial (GFAP) lineage marker, brain tumor stem cell markers (Nestin and Sox2), and human group 3 MB markers (LGR5 and NPR3).

Subtask 5: tumor gene expression profiling using microarray. (Month: 11-13)
We have extracted RNAs from tumor tissues derived from Atoh1+ NPCs with the transfection of MYC+DNp53 (n=2). These RNAs have been subjected to Affymetrics microarray analysis for gene expression profiling. Our biostatistician Dr. Hongkai Ji is analyzing these microarray data and comparing them with human WNT, SHH, Group 3 and 4 MB subtypes.

Milestone achieved: We have successfully established MYC-driven MB tumors from iPSC-derived NPCs. We have also extensively characterized these tumors by using histopathological and molecular endpoints to confirm that these tumors recapitulate human group 3 MB. More importantly, we successfully established neurosphere cultures from these MYC-driven MB tumors. This cell model will greatly facilitate in vitro mechanistic studies and large-scale drug screen for targeting cancer stem cells in MYC-driven MB. Overall, following the Statement of Work, we have achieved milestones of Subtask 1-4. We have also made progress in Subtask 5.

Major Task 3: Test the effects of enforced Atoh1 expression before and after MYC-driven MB tumors have formed.
Subtask 1: Perform cell infection and transplantation. (Month: 11-12)
We have confirmed Atoh1 induction in neurospheres derived from MYC-driven MB tumors. Atoh1 transgene in these neurospheres can be induced by Doxycycline (Fig. 5), thus allowing us to further study the effects of enforced Atoh1 expression before and after MYC-driven MB tumors have formed. We have transplanted these cells into the brains of immunodeficient mice for MB tumor formation and Doxycycline treatment.
Other experiments proposed for Subtask 2-4 described in the Statement of Work are in progress.

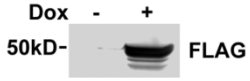

Fig. 5 Neurospheres derived from MYC-driven MB tumors were treated +/- Doxycyline for 48 hours. Transgenic Atoh1 (with FLAG tag) expression were detected by immunoblotting for FLAG.

Specific Aim 2: To identify the genome-wide MYC-regulated transcriptional network responsible for MYC-driven tumorigenesis, and to determine the effects of Atoh1 expression on MYC-regulated transcriptional events.
Subtask 1: perform MYC ChIP-seq in four different cell contexts (Months: 6-10)
Subtask 2: perform RNA-seq in four different cell contexts (Months: 9-12)
In order to optimize the protocol for MYC ChIP suitable for our cell models, we have performed MYC ChIP on neurospheres isolated from MYC-driven MB tumors. Our initial experiments are not successful because we cannot collect enough cells for the ChIP experiment that requires large amount of cells. However, we have solved this problem by using neurosphere cultures from MYC-driven MB tumors that can be efficiently expanded in vitro (Fig. 3A). We have used MYC western blotting to confirm the immunoprecipitation efficiency of MYC ChIP, and we found that ChIP using MYC antibody efficiently precipitates MYC proteins from these neurospheres. To ensure the quality of our ChIP-seq samples, we are currently performing ChIP-PCR to confirm the binding of MYC to promoters of known MYC target genes, such as Prps1, Gart and Pfas. After finishing these experiments, we will send these MYC ChIP-seq libraries for high-throughput sequencing. We have also prepared RNA-seq libraries for identifying differentially expressed genes after MYC induction. We are performing RT-PCR to ensure that know MYC-activated genes (e.g. Prps1, Gart and Pfas) are upregulated in response to MYC induction. After these experiments , we will send these RNA-seq libraries to our high-throughput sequencing facility in July 2015.
Milestone achieved: Although we encountered a minor problem when performing Subtask 1, we have used an alternative cell culture strategy to solve this problem. Experiments for Subtask 1 and 2 are currently in progress. We anticipate that the milestones of these two tasks will be achieved during August 2015.

Training and Career Development Tasks:
Major Task 1: Training in analyzing MB tumors (Month: 1-12)
Subtask 1: Learn the principles of MB pathology
Subtask 2: Learn the pathology of four MB subtypes
Under the mentorship of Dr. Eberhart, I have learned the principles of MB pathology and the pathology of MB subtypes. We have reviewed together the pathological features of different MB subtypes.
Milestone achieved: I am now able to independently perform pathological analysis on MYC-driven MB tumors, based on the pathological features of this MB subtype, such as poorly differentiated, medium to large size cells with nuclear molding and prominent nucleoli, and numerous mitotic and apoptotic tumor cells.

Major Task 3: Career development in brain tumor research
Subtask 1: Attend a grant writing seminar
I have attend American Association for Cancer Research Annual Meeting 2015 and also American Brain Tumor Association Alumni Research Network Meeting 2014. I have joined grant writing seminars during these meetings and imporved my grant writing skill.
Subtask 2: Presentation at quarterly department brain tumor seminar
I have present my research in March 2014 at the Neuro-Oncology Research Meeting from Neurology department.
Subtask 3: Presentation at American Association for Cancer Research and Society for Neuro-Oncology annual meeting (2014 and 2015)
I gave an oral presentation in a Minisymposium session (Mouse Models of Human Cancer 2) at the 2015 American Association for Cancer Research Annual Meeting (also see Appendices for abstract of this presentation). I am also invited to write a review paper for the journal Pharmacology & Therapeutics focusing on medulloblastoma and medulloblastoma models (see Appendices for the invitation)
Milestone achieved: I have successfully present data from this project at a national meeting. I will attend more cancer research meetings to present my research, establish new collaborations, and improve my grant writing skill.

How were the results disseminated to communities of interest?
Nothing to report.

Plan for the next reporting period:
Our current progress matches the milestones proposed in the Statement of Work. For the next reporting period, we will continuously perform our proposed experiments in Specific Aim 1&2 to achieve our proposed milestones. I will also get training in analyzing MB tumors, and microarray and ChIP-seq data. Overall, we are looking forward to submitting one or two manuscripts to publish results from this project, and also submitting one or two grant applications for continuous research on pediatric brain tumors.

Impact:

Impact on the development of the principal discipline of the project:
The principal discipline of this project is to model MYC-driven medulloblastoma by using human stem cells and to further study the transcriptional mechanisms underlying the tumor formation process of MYC-driven medulloblastoma. Our current results strongly support that neural progenitors induced from human induced pluripotent stem cells by Atoh1 induction can be efficiently transformed by MYC oncogene to form aggressive brain tumors that recapitulate human group 3 medulloblastoma. We further show that it is feasible to establish neurosphere cultures from these medulloblastoma tumors to enrich brain tumor stem cells. This neurosphere culture model are suitable for studying gene functions and also testing novel therapies. Overall, this new MYC-driven medulloblastoma model we established provides a reliable model for developing and testing potential therapies for this highly aggressive pediatric brain tumors. It is also feasible to use this medulloblastoma model derived from human stem cells to perform personalized drug discovery and investigate the role of individual's distinctive genetic background in carcinogen sensitivity and medulloblastoma susceptibility.

Impact on other disciplines: nothing to report.

Impact on technology transfer: nothing to report.

Impact on society beyond science and technology: nothing to report.

Changes/Problems:

We encountered a minor problem when we performed Subtask 1 of Specific Aim 2. Our initial experiments are not successful because we cannot collect enough cells for the ChIP experiment that requires large amount of cells. However, we have solved this problem by using neurosphere cultures from MYC-driven MB tumors that can be efficiently expanded in vitro (Fig. 3A). This problem only caused a delay of 3 months for Subtask 1 of Specific Aim 2, and we anticipate that the milestones of this subtask will be achieved in the next two months.

Products:

Conference presentation:
A MYC-driven medulloblastoma model derived from human induced pluripotent stem cells.
Jonathan Sagal[1], Charles G. Eberhart[2], Mingyao Ying[1].
[1]Kennedy Krieger Research Institute, Baltimore, MD;
[2]Johns Hopkins University School of Medicine, Baltimore, MD
Oral presentation in Mouse Models of Human Cancer 2 minisymposium session.
2015 American Association for Cancer Research Annual Meeting
Monday, Apr 20, 2015

Participants & Other Collaborating Organizations:

Name: Mingyao Ying
Project Role: Principle investigator
Nearest person month worked: 4.8
Contribution to Project: Dr. Ying is responsible for supervising and finishing all the proposed experiments, and analyzing data and preparing publication.
Funding Support: NIH

Name: Sagal Chaim
Project Role: Lab technician
Nearest person month worked: 3.6
Contribution to Project: Sagal Chaim collaborates with Dr. Ying to run the experiments and analyze data.

Name: Charles Eberhart
Project Role: Designated Collaborator
Nearest person month worked: 1.2
Contribution to Project: Dr. Eberhart serve as Dr. Ying's mentor in the project and perform pathological analyses of medulloblastoma tumors.

Special Reporting:

None

Appendices:

Invitation letter for writing a review focused on medulloblastoma and medulloblastoma models.
Abstract for 2015 American Association for Cancer Research Annual Meeting.

Invitation to Contribute

Teicher, Beverly (NIH/NCI) [E] [beverly.teicher@nih.gov]

Sent: Tuesday, May 26, 2015 11:03 AM
To: Ying, Mingyao
Attachments: AuthorsInformationP&TMay2010.doc (54 KB)

Dr. Mingyao Ying
Kennedy Krieger Research Institute
Room 424
707 N. Broadway
Baltimore, MD 21205

Dear Dr. Ying,

I am writing to you as an Executive editor for the journal Pharmacology & Therapeutics. Pharmacology & Therapeutics is a review journal which seeks manuscripts that are critical, authoritative reviews on timely topics. This journal has a strong presence in oncology research and has a 5-year impact factor of 9.3.

Because of your standing and expertise in the field, I extend an invitation to you to prepare a review on the topic: medulloblastoma and medulloblastoma models. The instructions to authors are attached. Of course, you can choose co-authors and focus the review as you wish. The timeline for completion of the manuscript would be 9 months.

I hope that you are interested in pursuing this project.

Best Regards,
Beverly A. Teicher

Beverly A. Teicher, PhD
Chief, Molecular Pharmacology Branch
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Email: teicherba@mail.nih.gov

Confirmation of P&T #22837

Jennifer McNichols [jmcnichols@kumc.edu] on behalf of P&T [P&T@kumc.edu]

Sent: Wednesday, May 27, 2015 4:27 PM
To: Ying, Mingyao
Cc: beverly.teicher@nih.gov
Attachments: P&TAuthorsInformationNovem~1.doc (50 KB)

Dear Dr. Ying,

We are pleased to learn from Dr. Teicher that you have agreed to author an article with the working title of "Medulloblastoma, models to recapitulate different subtypes of medulloblastoma and their pre-clinical application" for publication in Pharmacology & Therapeutics. Your manuscript is scheduled for submission by February 02, 2015 to Dr. Teicher (via beverly.teicher@nih.gov). Attached is a Word document containing the Author Instructions. Please alert me if you encounter any problems opening the attachment or have questions.

To thank you for writing this article for P&T we are pleased to offer you 3 months free access to Scopus. It is the world's largest abstract and citation database that offers access to more than 15,000 peer-reviewed titles from more than 4,000 international publishers. If you would like to accept this offer then please let me know and I will forward your request to the Elsevier Office, who will then send you a username and password. You may be contacted at the conclusion of the complimentary subscription for your feedback on the service.

Thank you!

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Editorial Coordinator

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Tuesday, July 07, 2015

Re: AACR Annual Meeting 2015 in Philadelphia, PA
Abstract Control Number: 1672
Title: A MYC-driven medulloblastoma model derived from human induced pluripotent stem cells

Dear Dr. Ying:

Your above-referenced abstract has been scheduled for oral presentation in a Minisymposium session at the 2015 AACR Annual Meeting in Philadelphia, PA and will be published in the 2015 Proceedings of the American Association for Cancer Research. Presentation information pertaining to your abstract is below:

Session Category: Tumor Biology 1

Session Date and Time: Monday Apr 20, 2015 3:00 PM - 5:00 PM

Permanent Abstract Number: 2892

Please refer to the printed Final Program [distributed onsite] or the online Annual Meeting Itinerary Planner [available in late February through the AACR Website at http://www.aacr.org] for the exact location of your presentation.

Instructions for Presenters in Minisymposia are available through the 2015 AACR Annual Meeting website. Please visit the website for more information on oral presentations.

Minisymposium presenters at the AACR Annual Meeting must register for the full meeting at the rate appropriate to their membership status and obtain their own hotel accommodations. Registration and housing information are included below:

Advance Registration Deadline: February 9, 2015
Online Registration and Letters of Invitation

Special Offer for Non-Member Presenters
If you are not currently a member of the AACR, there is still time to join and enjoy significant savings

on your registration for the 2015 AACR Annual Meeting. Individuals who are interested in joining the AACR and registering at the advance member rate must submit a membership application no later than 5:00 PM ET Friday, February 6. Interested candidates can apply for membership online at http://myaacr.aacr.org/Default.aspx. If you have any questions or need further information on becoming an AACR member, please contact the AACR Member Services Department at (215) 440-9300 or membership@aacr.org.

Housing Deadline: March 4, 2015
Online Housing System

Travel Information
[Airline/Train Reservations; Ground Transportation;
Official Letters of Invitation for International Attendees]

For more information, visit the AACR Annual Meeting 2015 home page.

Thank you for your participation in the 2015 AACR Annual Meeting.

Sincerely,
Lewis C.Cantley, Ph.D.
Program Committee Chairperson

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Status: Completed
WebcastSelectionNotification: Agree to participate including all slides
WebcastSignature: ying@kennedykrieger.org

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Presentation Abstract

Abstract Number:	2892
Presentation Title:	A MYC-driven medulloblastoma model derived from human induced pluripotent stem cells
Presentation Time:	Monday, Apr 20, 2015, 3:35 PM - 3:50 PM
Location:	Room 121, Pennsylvania Convention Center
Author Block:	Jonathan Sagal[1], Charles G. Eberhart[2], Mingyao Ying[1]. [1]Kennedy Krieger Research Institute, Baltimore, MD; [2]Johns Hopkins University School of Medicine, Baltimore, MD
Abstract Body:	Brain tumors are the most common cause of childhood oncological death, and medulloblastoma (MB) is the most common malignant pediatric brain tumor. Current MB treatments yield five-year survival rates of 60-70%, but usually result in significant neurological, intellectual and physical disabilities. Recent gene expression studies have identified four MB subgroups, many of which have unique clinical and histopathological features. Patients with Group 3 MB are more likely to have aggressive and invasive tumors with large cell/anaplastic histology, and have the worst prognosis. Group 3 MB is characterized by amplification and overexpression oncogenic transcription factor MYC, herein referred to as MYC-driven MB. Modeling MYC-driven MB is critical for developing and testing potential therapies for this highly aggressive MB. Recently, murine MYC-driven MB models have been developed using mouse neural stem cells (NSCs) or neuronal precursor cells (NPCs). But human MB models derived from individual-specific cells are still lacking. Human induced pluripotent stem cells (iPSCs) can be differentiated into various types of cells and hold great promise for developing individual-specific disease models. It

is valuable to develop MB models using human iPSCs from both MB patients and unaffected persons. In comparison with mouse-cell-derived MB models, human-iPSC-derived MB models will provide a unique and high-impact platform not only for personalized drug discovery but also for studying the role of individual's distinctive genetic background in carcinogen sensitivity and MB susceptibility. Transcription factor Atoh1 governs the development of cerebellar granule neurons and is essential for MB formation. Here, we induced Atoh1 in human iPSCs to differentiate these cells into NPCs. We further infected these Atoh1-induced NPCs with lentiviruses encoding a stabilized form of MYC (MYCT58A) and dominant-negative p53 (DNp53). These NPCs generated aggressive tumors after being transplanted into mouse cerebellum. NPCs infected by DNp53 alone did not form tumors after 90 days. These MYC-driven tumors were comprised of poorly differentiated, medium to large size cells which showed nuclear molding, prominent nucleoli and numerous mitotic (Ki67+) and apoptotic (Cleaved-Caspase-3+) cells. These tumors also expressed early neuronal lineage marker (β-tubulin III). All these features closely mimic human Group 3 MB. Moreover, we also established neurosphere cultures from these MYC-driven tumors to enrich cancer stem-like cells that have the capability for long-term self-renewal and tumor initiation upon serial transplantations. In summary, we established a novel human-iPSC-drived cancer model for modeling MYC-driven MB. Our results support the feasibility to recapitulate human cancers using progenies derived from human iPSCs. The iPSC-derived MB model we established will facilitate mechanistic studies and drug testing for human aggressive MB.

American Association for Cancer Research
615 Chestnut St. 17th Floor
Philadelphia, PA 19106